PB-229 430

A METHOD FOR THE ISOLATION AND ENUMERATION OF ACTINOMYCETES RELATED TO WATER SUPPLIES

Robert S. Safferman, et al

Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio

1962

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

PB 229 430

SEC TR W62-10

A METHOD FOR THE ISOLATION AND ENUMERATION OF ACTINOMYCETES RELATED TO WATER SUPPLIES

Robert S. Safferman and Mary-Ellen Morris
Research Branch
Aquatic Biology Section

U. S. Department of Health, Education and Welfare
Public Health Service
Division of Water Supply and Pollution Control

Robert A. Taft Sanitary Engineering Center
Cincinnati 26, Ohio

1962

19

CENTER PUBLICATIONS

The Robert A. Taft Sanitary Engineering Center is a national laboratory of the Public Health Service for research, training, and technical consultation in problems of water and waste treatment, milk and food safety, air pollution control, and radiological health. Its technical reports and papers are available without charge to professional users in government, education, and industry. Lists of publications in selected fields may be obtained on request to the Director, Robert A. Taft Sanitary Engineering Center, Public Health Service, Cincinnati 26, Ohio.

CONTENTS

	Page
Introduction	1
General Discussion	1
Materials	3
Preliminary Preparations	3
Collection of Samples	4
Preparation of Samples	5
Preparation of Plates	6
Identification of Actinomycete Colonies	7
Counting Procedure	12
Calculation Procedure	12
Disposal Procedure	13
Selected References	14

A METHOD FOR THE ISOLATION AND ENUMERATION OF ACTINOMYCETES RELATED TO WATER SUPPLIES

INTRODUCTION

In recent years considerable attention has been given to evaluating the relationships between actinomycete development and the earthy taste and odor problems frequently encountered in potable waters. Increased concern over these problems has resulted in extensive inquiries concerning the isolation and enumeration of those actinomycete groups which may significantly affect water quality. Although there are several excellent publications on the subject, they are highly technical and do not emphasize a basic approach to the problems. In an effort to help fill this need, procedures are set forth here in considerable detail so that they can be adopted by investigators who have had little experience with this group of organisms. The methods described are well established techniques that have been employed with considerable success by the authors. In several instances it has been necessary to clarify these procedures with supplementary material. Specific techniques are suggested where it is not possible to refer to any single procedure.

GENERAL DISCUSSION

Since the actinomycetes are generally considered to represent an intermediate stage between the bacteria and the filamentous fungi, it is not surprising that they are often confused with these particular microorganisms. A thorough review of their characteristics, however, leaves little doubt that the actinomycetes are more closely related to the bacterial form. On this basis, the organisms have been placed in "Bergey's Manual of Determinative Bacteriology" under the order *Actinomycetales*. According to Waksman (1961), this order is comprised of three families that are further subdivided into ten genera containing about 350 species. In view of their ecological distribution and general nature, only

three of the ten genera *(Streptomyces, Nocardia,* and *Micromonospora)* are likely to include species which are of consequence in water supply problems.

Members of these three genera can generally be differentiated according to the characteristics outlined in Table 1. A generic problem does exist however, in the classification of certain strains of the *Streptomyces–Nocardia* group. This predicament is particularly evident in those forms of the genus *Streptomyces* which, on continued cultivation, have lost their capacity to form aerial mycelia.

Table 1. Differential Characteristics of the Genera *Nocardia, Micromonospora,* and *Streptomyces.*

Genera	Substrate mycelium	Aerial mycelium	Conidia formation
Nocardia	Hypha fragments into bacillary and coccoid forms	Little or none	None
Micromonospora	Hypha non-fragmenting	Little or none	Singly on short sporophores
Streptomyces	Hypha non-fragmenting	Abundant	In chains on aerial mycelium

The actinomycetes are most commonly represented by saprophytic forms that are essential in the decomposition and transformation of complex organic residues. In natural processes, therefore, these organisms constitute a vital factor in sustaining an ecologically balanced system. Though actinomycetes are widely distributed in nature, it should be noted that in soils and in lake and river muds they comprise a considerable proportion of the population. Furthermore, in these habitats members of this group undoubtedly have the capacity to produce odoriferous substances in quantities that can be detected in the immediate environment. Of the three genera previously discussed, the genus *Micromonospora* is usually associated with lake and river muds, whereas the *Streptomyces* and *Nocardia* forms are abundant in soil habitats.

In the reports available on earthy tastes and odors in potable water systems, it has been generally assumed that these adverse effects can be attributed to metabolites formed during normal development of the actinomycetes. However, marked differences of opinion have developed among those investigators who have studied this particular relationship. These differences are concerned primarily with the generic groups and ecological sites involved in the possible formation of the odoriferous materials. Before any definite conclusions can be made on these issues, extensive physiological investigations must be pursued under both field and laboratory conditions.

MATERIALS

Glassware

Petri culture dishes, 150 mm in diameter.
Measuring pipettes (Mohr type), 1, 2, 5, and 10 ml.
Volumetric pipettes, 15 ml.
Graduated cylinders, 25, 30, 100, 250, and 1000 ml.
Erlenmeyer flasks, 500 and 2000 ml.
Screw-cap bottles, 150 ml.
Bacteriological test tubes,
150 mm in length, 25 mm in diameter.
Ground glass stoppered bottles, 300 ml.
High temperature, wide mouth polyethylene bottles, 8 oz.

All glassware should be heat and chemical resistant.

Laboratory apparatus

Torsion balance graduated to 1 gm in 10 mg divisions.
Balance weights, 10 mg to 50 gm.
Bunsen gas burner (alcohol burner may be substituted).
Constant temperature water bath.
Soil sampling tube.
Water sampler.

Media

Egg albumin agar:

Glucose,	1.0 gm;
Soluble egg albumin,	0.25 gm;
K_2HPO_4,	0.5 gm;
$MgSO_4.7H_2O$,	0.2 gm;
$Fe_2(SO_4)_3.7H_2O$,	0.01 gm;
Bacto-agar,	15.0 gm;
Distilled water,	1000 ml.

pH 7.1 after sterilization.

Sodium caseinate agar:

Glucose,	1.0 gm;
Sodium caseinate,	2.0 gm;
K_2HPO_4,	0.2 gm;
$MgSO_4.7H_2O$,	0.2 gm;
$FeSO_4.7H_2O$,	0.01 gm;
Bacto-agar,	15.0 gm;
Distilled water,	1000 ml.

pH 7.3 after sterilization.

PRELIMINARY PREPARATIONS

Particular attention should be given to the preparation of the materials used in these studies as carelessness can markedly affect the accuracy of the final results. For example, if 90-ml dilution bottles were erroneously prepared with only 85 ml of water, a 26 percent error would be introduced into those colony counts in which samples had been serially diluted at ten-fold intervals to a 1:1,000,000 dilution.

The egg albumin and sodium caseinate media are prepared by carefully weighing each of the nutrients and dissolving each, in turn, as it is added to a 2000-ml Erlenmeyer flask containing 1000 ml of distilled water. After the agar is introduced into the flask, the medium is heated by placing the flask in a container of boiling water, in order to prevent agar granules from burning or sticking to the vessel. As soon as the agar is dissolved, the medium is dispensed into 500-ml Erlenmeyer flasks (250-ml aliquots) and 50-ml test tubes (17-ml aliquots). The flasks and test tubes are then plugged with non-absorbent cotton and their contents labelled.

The water dilution blanks are prepared in 150-ml screw-cap bottles to which 90 ml of tap water are added. It should be noted that for dilution of microbial populations tap water is preferred to distilled water, as plasmolysis occurs, when the latter is used. The bottles are loosely capped to avoid breakage from excessive vapor pressure produced during sterilization.

In order to suppress fungal development on the isolation plates, Actidione*, an antifungal antibiotic, is added to the medium just prior to plating out the sample. For this purpose, a stock solution of the antibiotic is prepared at a concentration of 1 mg per ml of distilled water.

The culture media and water blanks are sterilized at 15 psi steam pressure (121° C) for 20 minutes and the Actidione stock solution for 15 minutes. Although glassware, such as pipettes and petri dishes can be autoclaved, dry heat sterilization at 170° C for one hour is recommended. Prior to sterilization petri dishes and pipettes should be either wrapped in brown paper or placed in cannisters.

COLLECTION OF SAMPLES

Since the samples form the basis for all subsequent correlations between actinomycete development and earthy taste and odor problems, any marked deviation in the procedure can be expected to produce erroneous data. At the outset of such studies, therefore, it is essential that the techniques and locations to be used in the sampling of an area be carefully evaluated by preliminary experiments on the affected system.

To determine the ecological relationships between actinomycete development and earthy odor problems, it is suggested that the following sampling locations and procedures be employed.

Water sampling sites:

A. Raw water
   1. Surface water in midstream or midlake.
   2. Surface water along banks.
   3. Water 6 to 12 inches above bottom sediment at midstream or midlake.

* Produced by Upjohn and Co., Kalamazoo, Michigan.

B. Water purification plant
   1. Raw water intake prior to any treatment.
   2. Finished water just before entering the distribution lines.
   3. Water in the distribution lines.

Water samples are collected in sterile 300-ml ground glass stoppered bottles that are lowered upside down 1 to 3 inches beneath the surface and then carefully turned over to avoid floating debris. Bottom water samples, in most cases, will require the use of a water sampler.

Soil sampling sites:
1. Bottom sediment at midstream or midlake at a depth of 1 to 6 inches.
2. Bank soil at water line.
3. Soil beyond flood crest.
4. Areas rich in organic matter, such as marshlands or mud flats.

The very top layer of soil is scraped off with a clean spatula and soil 1 to 3 inches beneath the surface is placed in a sterile wide-mouth polyethylene bottle. Samples from the bottom sediment are obtained with a soil sampling tube. In collecting the samples, absolute sterility is not required since contaminants introduced during this process are generally insignificant.

To reduce variation in actinomycete counts, four soil or water composites of at least five samples each should be made at each sampling site. In addition, samples should be plated out as soon after collection as possible. If it is necessary to store samples, they should be kept at 3° to 5° C for not more than 24 hours.

PREPARATION OF SAMPLES

Soil

Prior to weighing the test sample, the collected soil is thoroughly mixed with a clean spatula and its volume measured. To carry out this latter technique, a 10-gm aliquot of the soil sample is added to a 50-ml graduated cylinder containing 30 ml of water, and the soil volume determined by measuring the amount of water displaced in the cylinder.

The initial dilution is prepared by placing 10 gm of the soil sample in a 150-ml bottle containing 90 ml of sterile tap water and aseptically adding the quantity of water needed to give a total volume of 100 ml. The suspension is shaken vigorously either by

hand or on a mechanical shaker for 3 minutes. Before settling occurs, 10 ml of the above 1:10 soil dilution are pipetted into another 150-ml bottle containing 90 ml sterile tap water and shaken vigorously for 15 seconds to give a 1:100 dilution. A 10-ml aliquot is then immediately withdrawn, transferred into a 90-ml water dilution bottle and shaken to give a 1:1000 dilution. The process is repeated until a final dilution of 1:1,000,000 is reached. This ten-fold dilution series generally includes a set of plates in which the total count ranges from 30 to 300 colonies. Following several examinations of a sampling area, the procedure can often be simplified by reducing the number of dilutions employed in plating out a sample.

To avoid difficulties arising from variation in the moisture contents of sampled soils, a 50-gm aliquot of each soil is weighed on tared weighing pans and dried at 105° to 110° C to a constant weight. The weight of the dried soil is calculated and used in determining the number of actinomycetes per gram of dry soil.

Water

After a composite water sample is thoroughly mixed, a 10-ml aliquot is immediately withdrawn and transferred to a 90-ml sterile water dilution blank in a 150-ml bottle. Ten-fold serial dilutions are then made according to the procedure described for the soil samples. In most cases, however, dilutions up to 1:1000 are sufficient.

PREPARATION OF PLATES

Of the methods available for enumerating microbial populations, the plate count is generally considered the most reliable. The use of a solid medium permits formation of colonies which, in the case of the actinomycetes, are a mass of branching filaments originating from a spore or a mycelial fragment. To facilitate the identification and counting of actinomycete colonies, a two-layer agar plate has been adapted for these studies. As only the thin upper layer is inoculated, the technique assures a predominance of surface colonies.

The bottom, or basal layer consists of 15 ml of uninoculated medium. This is prepared by immersing a flask of sterile egg albumin agar (or sodium caseinate agar) in boiling water and then pipetting a 15-ml aliquot of the melted agar onto a sterile petri plate, opening the petri plate only long enough to introduce the agar. After the basal layer has solidified, 5 ml of inoculated agar is then introduced into the plate.

The surface layer is prepared in a test tube containing 17 ml of egg albumin agar (or sodium caseinate agar) that has been previously melted and cooled. As agar solidifies at 42° C, the medium is kept at 45° to 48° C in a constant-temperature water bath. Under no circumstances should a sample be inoculated into a medium with a temperature higher than 50° C. A 2.0-ml aliquot of the appropriate diluted sample and 1.0 ml of the Actidione stock

solution (1 mg/ml) are added to the test tube. This results in a final volume of 20 ml and an Actidione concentration of 0.05 mg per ml of medium. When pipetting 5 ml of the inoculated medium over the basal layer of the plate, the medium should be gently swirled so that an even distribution of the surface agar layer is obtained. Dilutions made from each of the composite samples are plated out in triplicate and their average actinomycete count determined.

Plates are incubated at 20° to 30° C until no new colonies appear. The time necessary for development of the colonies depends largely on the culture medium, the origin of the sample, and the temperature of incubation. The authors have found that most egg albumin and sodium caseinate agar plates can be counted following a 6- to 7-day incubation period at 25° C. Nevertheless, it is advisable to retain all plates for at least 14 days.

IDENTIFICATION OF ACTINOMYCETE COLONIES

Because of similarities in the gross appearance of actinomycete and bacterial colonies, researchers with little experience in the identification of actinomycetes often find it difficult to differentiate visually between these two groups. Microscopic examination of the actinomycete colonies will quickly verify their identity; the general use of this procedure however, is much too cumbersome and tedious when numerous plate counts are involved. Several of the distinguishing characteristics commonly employed in the macroscopic differentiation of actinomycete and bacterial colonies are summarized in Table 2. Differences in the macroscopic appearance of these organisms on isolation plates are also illustrated in Figure 1.

Table 2 Comparison of Actinomycete and Bacterial Colonies on Solid Medium

Characteristics	Bacterial Colony	Actinomycete colony
Appearance	Shiny or opalescent	Dull, and chalky when colony covered with aerial mycelium
Texture	Soft	Tough and leathery
Degree of adherence to solid medium	Weak	Strong
Edge of colony:		
Macroscopic	Generally no distinction from the colony as a whole	Appears less dense, producing a halo effect
Microscopic (X 100)	Smooth	Fuzzy

Addition of Actidione to the isolation media suppresses development of fungal colonies in the soil and water dilution plates. Those that do develop can be readily differentiated from the actinomycetes by their wooly appearance and considerably larger cell diameter. On a solid medium, actinomycete mycelia generally have a diameter of 0.5 to 1.0 micron, whereas the fungal filaments range from 2 to 10 microns.

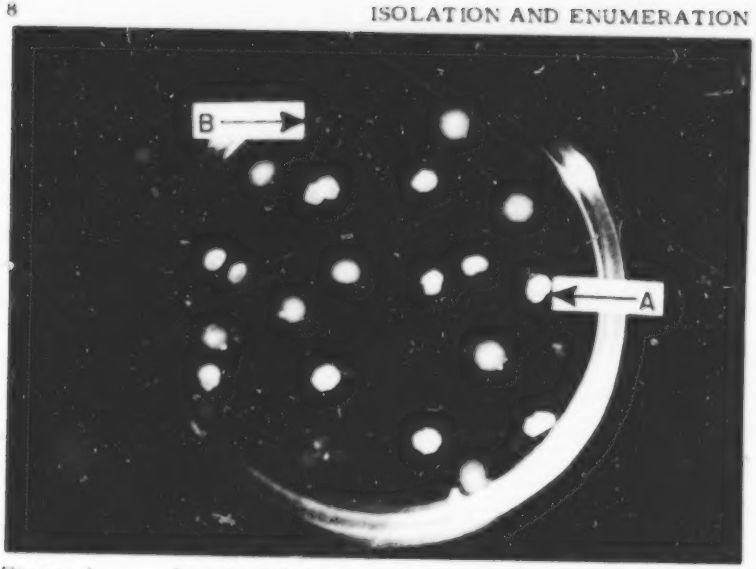

Figure 1. An egg albumin isolation plate showing bacterial and actinomycete colonies. "A", a sporulating actinomycete colony; "B", a bacterial colony. Note that actinomycete colonies are characterized by a dull and powdery appearance in contrast to the smooth and mucoid appearance of the bacterial colonies.

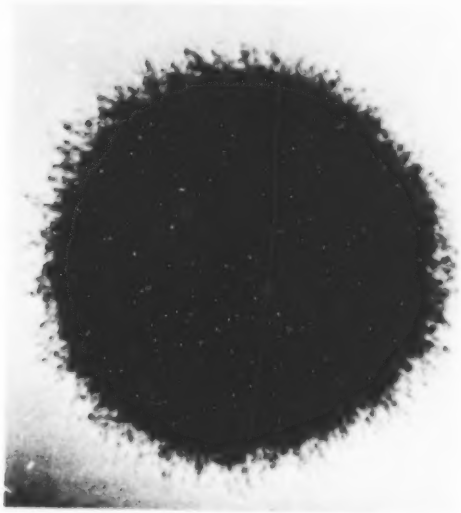

Figure 2. Actinomycete colony. (X 225)

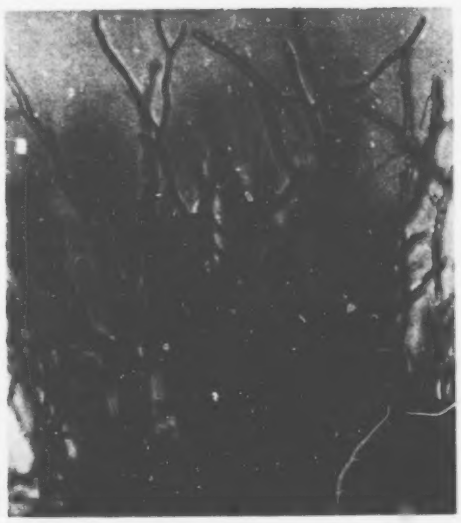

Figure 3. Portion of a fungal colony.
(X 225)

Figure 4. Bacterial colony.
(X 225)

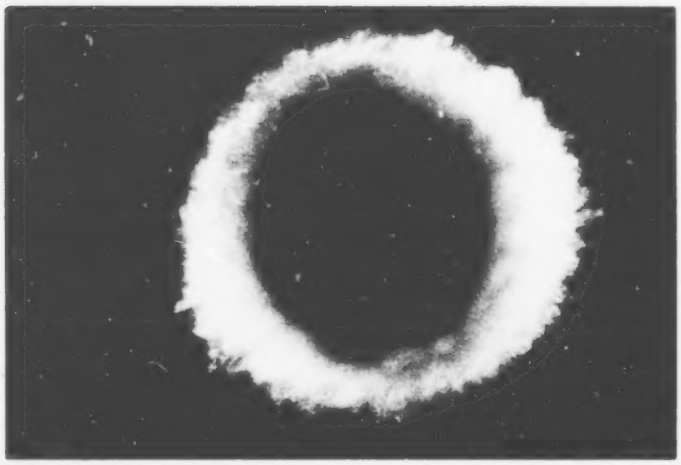

Figure 5. Actinomycete colony (phase microscopy). (X 225)

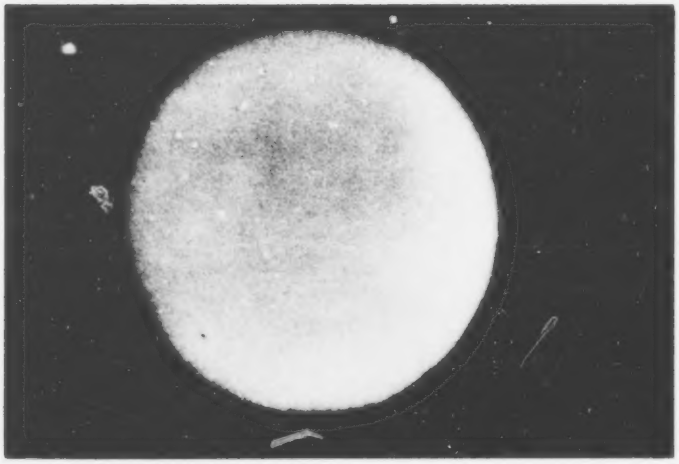

Figure 6. Bacterial colony (phase microscopy). (X 225)

Figure 7. Comparison of actinomycete (left), fungal (middle), and bacterial (right) colonies. (X 375)

Reproduced from best available copy.

Marked distinctions in the general appearance of actinomycete, bacterial, and filamentous fungal colonies are evident in the photomicrographs shown in Figures 2 to 7. Particular attention should be given to the periphery of the actinomycete and of the bacterial colonies as it is an excellent means of differentiating the two groups when any doubt arises as to their macroscopic identification. The term "actinomycete" (derived from Greek: actino = ray, and mycete = fungus) aptly describes the radial arrangement of filaments found in the individual colonies. A detailed examination of Figures 2 and 5 shows that this ray-like appearance is due to the filamentous nature of the organism.

COUNTING PROCEDURE

Actinomycete counts are determined from those dilution plates in which the total number of colonies is between 30 and 300. Inasmuch as the preparation of these plates involves the use of samples that were serially diluted at ten-fold intervals, only one set of plates in each series will generally fall within the limits specified above. If in the same dilution series this should occur in more than one group, counts should be limited to the lower dilution.

A simple procedure for expediting macroscopic counts is marking off the bottom of a petri dish into quadrants and counting the actinomycetes in each quadrant by holding the plate in front of a light source, so that the actinomycete and bacterial differences are accentuated.

CALCULATION PROCEDURE

To determine the actinomycete population of a sampled area, the following calculations are carried out:

1. Average the actinomycete counts in each set of triplicate plates.

2. Multiply this average by the serial dilution number of the particular plate.

3. Multiply the product resulting from steps 1 and 2 by a factor of 4. This takes into account the fact that only 5-ml aliquots of the 20 ml inoculum were added to each plate (1:4 dilution).

The final product represents the number of actinomycetes per ml of samples water or per gm of sampled soil.

In general application of this data, the number of actinomycetes determined per ml of sampled water can be recorded for all practical purposes in terms of organisms per gm of water. For comparative purposes, soil actinomycete determinations should be based on the dried weight of the soil sample. This avoids substantial errors that may result from differences in the moisture content of the tested areas. To calculate the theoretical number of actinomycetes per gm of dried soil, the actinomycete counts determined in step 3 above are multiplied by a conversion factor that was determined by dividing 50 gm of the sampled soil by its dried weight.

For example, if plates which had been inoculated with a 1:1000 soil dilution had actinomycete colony counts of 120, 125, and 127, and a 50-gm aliquot of the same soil sample when dried was found to weigh 35 gm, then the actinomycete density would be calculated as follows:

124 (average count) x 1000 (serial dilution) x 4 (medium dilution) = 496,000 actinomycetes/gm sampled soil.

$$\frac{\text{50.0 gm (wt. soil sample)}}{\text{35.0 gm (wt. dried soil sample)}} = 1.43 \text{ (conversion factor)}$$

1.43 (conversion factor) x 496,000 (actinomycetes/gm sampled soil) = 709,000 actinomycetes/gm dried soil.

DISPOSAL PROCEDURE

All glassware that has been used in the isolation procedure should be sterilized before cleaning. This safety precaution should be observed, especially when working with unidentified organisms.

SELECTED REFERENCES

Adams, B.A. 1929. The *Cladothrix dichotoma* and allied organisms as the cause of an "indeterminate" taste in chlorinated water. Water and Water Eng. 31: 327-329.

Adams, B.A. 1931. The cause and removal of earthy tastes in water. Water and Water Eng. 33: 572-574.

American Public Health Association. 1960. Standard methods for the examination of water and wastewater. 11th ed. New York, N. Y.

Breed, R.S., E.G.D. Murray, and N.R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed. Williams and Wilkins, Baltimore, Md.

Corke, C.T., and F.E. Chase. 1956. The selective enumeration of actinomycetes in the presence of large numbers of fungi. Canad. Jour. Microbiol. 2: 12-16.

Erikson, D. 1941. Studies on some lake-mud strains of *Micromonospora* Jour. Bacteriol. 41: 277-300.

Ferramola, R. 1949. Earthy odors produced by *Streptomyces* in water. Jour. Inter-American Assoc. San. Eng. 2: 358-380.

Issatchenko, B., and A.A. Egorova. 1944. Actinomycetes in reservoirs as one of the causes responsible for the earthy smell of their waters. Microbiologiya (U.S.S.R.) 13: 216-225.

Pease, D. 1951. Some simple procedures in sterile technique. Edwards Brothers, Ann Arbor, Michigan.

Porter, J.N., J.J. Wilhelm, and H.D. Tresner. 1960. Method for the preferential isolation of actinomycetes from soils. Appl. Microbiol. 8: 174-178.

Romano, A.H. 1958. Identification of odors produced by actinomycetes. Public Works. 89 (12): 100-101.

Silvey, J.K.G., J.C. Russell, D.R. Redden, and W.C. McCormick. 1950. Actinomycetes and common tastes and odors. Jour. Amer. Water Works Assoc. 42: 1018-1026.

GPO 820678-2

Thaysen, A.C. 1936. The origin of an earthy or muddy taint in fish. I. The nature and isolation of the taint. Ann. Appl. Biol. 23: 99-104.

Umbreit, W.W. and E. McCoy. 1941. The occurrence of actinomycetes of the genus *Micromonospora* in inland lakes. Symposium on Hydrobiol. Univ. Wisconsin. p. 106-114.

Waksman, S.A. 1932. Principles of soil microbiology. 2nd ed. Williams and Wilkins, Baltimore, Md.

Waksman, S.A. 1950. The actinomycetes; their nature, occurrence, activities, and importance. Chronica Botanica, Waltham, Mass.

Waksman, S.A. 1959. The actinomycetes. I. Nature, occurrence, and activities. Williams and Wilkins, Baltimore, Md.

Waksman, S.A. 1961. The actinomycetes. II. Classification, identification and descriptions of genera and species. Williams and Wilkins, Baltimore, Md.

Waksman, S.A. 1922. Microbiological analysis of soil as an index of soil fertility: I. The mathematical interpretation of numbers of microorganisms in the soil. Soil Sci. 14: 81-101.

Waksman, S.A. 1922. Microbiological analysis of soil as an index of soil fertility: II. Methods of the study of numbers of microorganisms in the soil. Soil Sci. 14: 283-298.

GPO 820678-1